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<b>(21) International Application Number:</b> PCT/AU87/00199 <b>(22) International Filing Date:</b> 3 July 1987 (03.07.87)  <b>(31) Priority Application Numbers:</b> PH 6721 PH 9205 <b>(32) Priority Dates:</b> 3 July 1986 (03.07.86) 27 November 1986 (27.11.86) <b>(33) Priority Country:</b> AU  <b>(71) Applicant (for all designated States except US):</b> THE STATE OF VICTORIA [AU/AU]; Treasury Place, Melbourne, VIC 3000 (AU).  <b>(72) Inventors; and</b> <b>(75) Inventors/Applicants (for US only) :</b> TILBROOK, Alan, John [AU/AU]; 20 Ormond Street, Kensington, VIC 3031 (AU). FAIRCLOUGH, Robert, John [NZ/AU]; 47 Pannam Drive, Werribee, VIC 3030 (AU).		<b>(74) Agents:</b> CORBETT, Terence, Guy et al.; Davies & Col- lison, 1 Little Collins Street, Melbourne, VIC 3000 (AU).  <b>(81) Designated States:</b> AT (European patent), AU, BE (Eu- ropean patent), CH (European patent), DE (Euro- pean patent), DK, FI, FR (European patent), GB (European patent), IT (European patent), JP, LU (European patent), NL (European patent), NO, SE (European patent), US.  <b>Published</b> <i>With international search report.</i>
<b>(54) Title:</b> COMPOSITION AND METHOD FOR IMMUNOLOGICAL CASTRATION AND SPAYING  <b>(57) Abstract</b>  Composition for the production in animals of antibodies specific for LHRH. The composition comprises two or more different carriers individually coupled to LHRH or analogues of LHRH, in amounts sufficient to elicit an immune response against LHRH. The compositions are useful for suppressing male social and sexual behaviour and oestrous cyclicity and ovulation in domestic animals.		

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COMPOSITION AND METHOD FOR IMMUNOLOGICAL CASTRATION  
AND SPAYING

The present invention relates to the suppression of social and sexual behaviour of male domestic animals and the suppression of ovulation and oestrous cyclicity of female domestic animals, and more specifically to a composition and method for immunological castration and spaying.

Normal sexual behaviour in male animals is maintained by androgens, or male sex hormones, these being produced mainly in the testes. One of the principal androgens is testosterone. It is common practice for farmers to eliminate sexual activity and to reduce aggressive behaviour of their stock by surgical castration. However, a number of disadvantages are associated with surgical castration. These include haemorrhage, infection and stress associated weight loss (Schanbacher, 1984). Furthermore, castration reduces both growth rate and the rate of lean tissue deposition (Robertson et al, 1970).

An alternative to surgical castration is active immunization against luteinizing hormone releasing hormone (hereinafter referred to as LHRH) or analogues thereof. LHRH is released from the hypothalamus into

the pituitary portal vessels. It has been suggested that LHRH binds to the gonadotrophs of the anterior pituitary to stimulate secretion and release of luteinizing hormone (LH). LH acts on the Leydig cells in the testis to increase the production of testosterone. Active immunization against LHRH disrupts the communication between the hypothalamus and pituitary. It is thought that the site at which antibodies to LHRH might inhibit reproductive function is in the hypophyseal portal blood vessels (Schanbacher, 1984).

Active immunization against LHRH leads to marked changes in the function and morphology of the anterior pituitary; this results in a reduction in the weight, gonadotrophin content and LHRH binding of the pituitary in several species including the rat (Kock, 1977; Fraser et al., 1982), rabbit (Arimura et al.; 1974) and monkey ((Chappel et al., 1980). In addition, there is a reduction in the secretion of testosterone and involution of the testes (rat: Fraser et al., 1982; marmoset monkey: Hodges and Hearn, 1977 ; ram: Schanbacher et al., 1982; Jeffcoate et al., 1982; bull: Robertson and others, 1979, 1981, 1982, 1984).

The rate of live weight gain in bulls actively immunized against LHRH is significantly greater than steers (Robertson et al., 1982). Immuno-castrates also convert food more efficiently, have a higher dressing out percentage, leaner carcasses and are shorter in height at the withers and thinner around the forgirth than steers (Robertson et al., 1982).

Bull calves that respond well to active immunization against LHRH show reduced secretion of gonadotrophins (Jeffcoate et al., 1982) and testosterone and a reduction in testicular size (Robertson and others, 1979, 1981, 1982, 1984). From subjective assessments of behaviour it appears that immuno-castrates resemble steers being more docile and easier to handle than bulls, (Robertson and others, 1979, 1981, 1984). When immunized bulls were permitted to serve an oestrous cow or an artificial vagina three out of the five animals that had high antibody titres did not attempt to mount while the other two attempted to mount and one of these achieved an ejaculation (Robertson et al., 1982). In an on-going study, Robertson et al. (1984) compared non-immunized bulls to animals that had responded well to immunization against LHRH and found that the immuno-castrates were less active than the bulls with regard to riding, heading and licking and moving.

Active immunization against LHRH has been reported to suppress ovulation and oestrous in female sheep (Clarke, Fraser and McNeilly, 1979) and cause gonadal atrophy in rabbits (Arimura et al., 1973), hamsters (de la Cruz et al., 1976) and rats (Fraser and Baker, 1978).

In the studies of Clarke et al., (1978) in sheep, Fraser et al. (1974) in rats and McCormack et al. (1977) in rhesus monkeys, anti-LHRH treatment resulted in a marked reduction in plasma LH concentrations. Thus it would appear that anti-LHRH treatment has a similar effect on plasma LH levels and gonad function

in females as in males. The study of Clarke et al. (1978) is particularly relevant as the ewes showing evidence of raised LHRH antibodies did become anovulatory and did not show oestrous cyclicity for two consecutive seasons.

Although active immunization against LHRH has been successfully achieved in bull calves and other animals, there has been considerable variation between individual animals in their response to immunization. In most studies, only about half the bull calves immunized against LHRH responded well to treatment having high antibody titres. This was reported for bull calves immunized at 12 weeks of age (Robertson and other, 1979, 1981), 20 weeks of age (Robertson et al., 1984) and 28 weeks of age (Robertson et al., 1982). Jeffcoate et al., (1982) actively immunized one month old bull calves against LHRH and reported that antibody titres rose after the animals had received two injections of immunogen but began to fall after a third injection. This necessitated a fourth injection to restore the antibody titres.

The phenomena of a lack of immune response to an antigen stimulus is well documented. For example in a text-book on Veterinary Immunology by Herbert (1976)) it is stated on pp. 199-200 that "In general, antibody titres found in members of a vaccinated population will be distributed on a statistical normal curve, a few animals having high titres and another small group failing to repond at all." In another section it is stated that "If a large enough number are tested (with antigen) some amongst those vaccinated will be found

to be unprotected. Errors of technique may account for a few of these but others may be quite incapable of achieving a state of protection immunity however many times the antigen is administered, or whatever other  
5 steps (i.e. the use of adjuvants) are taken to stimulate an immunity in them".

LHRH is a small peptide of 9 amino acids. In the field of immunology, it is well established that small immunogens elicit a poor immune response when injected  
10 into a host. Consequently, in each of the above studies, LHRH has been chemically conjugated to one particular carrier protein, for example, Human Serum Albumin.

The present applicants have surprisingly found that  
15 a synergistic interaction occurs when animals are immunised with a combination of two or more different carriers coupled to LHRH or analogues of LHRH. Combinations of LHRH or analogues of LHRH conjugated with carriers elicit an effective immune response  
20 (measured in terms of antibody titre) in a greater proportion of animals than if LHRH or analogues of LHRH conjugated to a single carrier are administered.

According to one aspect of the present invention there is provided a composition for the production in  
25 animals of antibodies specific for LHRH or analogues of LHRH, said composition comprising two or more different carriers individually coupled to LHRH or analogues of LHRH.

Analogues of LHRH are peptides where additions,  
30 deletions, modifications or alterations have been made to the natural sequence of LHRH. Alterations to the natural sequence include the addition of one or more amino acids to the amino or carboxy terminus of LHRH.

Domestic animal species falling within the scope of the invention include, cattle, sheep, goats, cats, guinea pigs, pigs, dogs, reindeer, horses and primates including women.

5 Carriers of any type which are useful immunogens may be utilised. Preferably the carriers are proteins and more preferably the proteins are soluble in aqueous solution although precipitated protein may be used.

Examples of preferred proteins are Diptheria Toxoid  
10 (DT), Human Serum Albumin (HSA), staphylococcus protein A (P.A.), Keyhole Limpet Haemocyanin (KLH), and Tetanus Toxoid, (TT).

Generally, any combination of two or more different carriers may be used. Preferably the carriers are  
15 proteins such as, HSA and P.A. or KLH and TT, each of these proteins being conjugated to LHRH. However, especially preferred are two or more carriers where each carrier coupled to LHRH and separately administered to an animal elicits a generally good titre response.  
20 Especially preferred are compositions where one of the carriers is Diptheria toxoid (DT).

Other carriers that may be used in the present invention include lipopolysaccharides, polysaccharides, glycopeptides, muramyl peptide analogues, peptidoclycans  
25 [from bacteria cell walls], liposomes, lecithin like materials, and bacterial proteins for example purified protein derivatives from tuberculin.

Conventional techniques may be used in conjugating LHRH and analogues of LHRH to various carriers. For  
30 example, heterobifunctional agents such as SPDP (Pharmacia, Piscataway New Jersey), carbodiimide, glutaraldehyde or biotin/avidin systems are useful. Conditions are generally selected so that LHRH or analogues of LHRH are maintained in its biological active conformation when coupled to the carrier.



Preferably the composition contains an adjuvant. Examples of adjuvants which may be used are aluminium hydroxide, Freund's incomplete adjuvant, Freund's complete adjuvant, DEAE dextran, levamisole, PCG and polyA, polyC  
5 or polyU. Especially preferred as adjuvants are materials that do not cause local inflammation. One example of such a material is a mineral oil composition that includes bacterial cell wall material such as peptidoglycans, or a synthetic derivative of such a cell  
10 wall material. Such a synthetic material is known as muramyl dipeptide.

The composition may be optionally buffered to physiological pH using buffers such as TRIS-HCl, HEPES, pipes or other appropriate buffers.

15 According to a further aspect of the present invention there is provided a method for the suppression of male social and sexual behaviour of domestic animals comprising the administration of a composition to a domestic animal said composition comprising two or more  
20 different carriers individually conjugated to LHRH or analogues of LHRH.

In an alternative aspect there is a method of suppressing the ovulation and oestrous cyclicity of female domestic animals by the administration of the  
25 above described compositions.

Administration may be parenterally, for example, subcutaneous, and/or intramuscular or intravenous injection, orally or by absorption through the skin or  
30 by a mini-pump either implanted in the animal or attached to the outside of the animal.

In the case of bovine, bull calves are preferably immunised with the composition at between 8 to 40 weeks of age. In the case of sheep, ram lambs are preferably immunised at 8 to 24 weeks. Booster injections are preferably given eight weeks apart and preferably 1 to 3  
booster injections are given.

In the previously defined method of the present invention, within the scope of the method is a treatment where the administration of the substance (e.g. LHRH) coupled to individual carriers is separate but the  
5 administration takes place over a relatively short space of time. A period of minutes or hours would usually work be satisfactory, although it would be obviously inconvenient compared to a single administration of the substance coupled to at least two carriers.

10 The present invention has particular utility in the immunological castration and spaying of domestic pets or companion animals, particularly cats and dogs. Conventional surgical treatments performed by veterinarians may be expensive, and often traumatic for  
15 the animal involved and its owner.

An increase in the proportion of animals responding may also occur where other substances that elicit an immune response, other than LHRH or analogues of LHRH, are conjugated to at least two different carriers and  
20 combinations of these conjugates are subsequently administered to animals.

Examples of such substances which elicit an immune response include peptides and proteins such as adrenocorticotrophic hormone, substance P, human  
25 chorionic gonadotrophin, somatostatin, epidermal and insulin-like growth factors; steroids including androstenedione (fecundin), testosterone and other substances such as melatonin and the prostaglandins.

In the descriptions the following terms have  
30 meanings set forth below:

LHRH: Luteinizing hormone releasing hormone is a specific substance involved in stimulating luteinizing hormone and follicle stimulating hormone from the adenohypophysis.

35 Carrier: Any substance, especially protein which, when coupled to small substances, can elicit an immune response to animals.

Antibody titre: The dilution of antisera which binds 50% of 0.15 nCi  $^{131}\text{I}$ -labelled LHRH for 16 h at  $4^{\circ}\text{C}$ . Precipitation of the gamma globulins was achieved using polyethylene glycol at a final concentration of 14.5%.

DT: Diphtheria toxoid.  
HSA: Human serum albumin.  
KLH: Keyhole limpet haemocyanin.  
10 TT: Tetanus toxoid.  
CP: Corynebacterium parvum.  
P.A.: Protein A from Staphylococcus aureus.  
Carb: 1-ethyl-3 (3-dimethyl aminopropyl) carbodiimide.  
15 Glut: Gluteraldehyde.

The present invention will now be described by way of example only with reference to the following experimental procedures, examples and tables.

20 Figures 1, 2 show Antibody Titre (Log Scale) plotted against various treatment groups for ram lambs and bull calves respectively. Figures 3 and 4 show mean testicular volume of ram lambs given Corynebacterium Parvum (CP), Keyhole Limpet Haemocyanin (KLH),  
25 Diphtheria Toxoid (DT), Tetanus Toxoid (TT) and a combination of these carriers coupled to LHRH; CP, KLH, DT in Figure 4 and KLH, TT and DT in Figure 3.

30 A. EXPERIMENTAL PROCEDURES

1. Preparation of LHRH conjugates

(a) Tetanus toxoid - LHRH

LHRH (240 mg) and tetanus toxoid (35.5 ml, 248 mg) was dissolved with 20 ml distilled water and the pH of the mixture adjusted to 6.0 with 0.1 N NaOH. A freshly prepared solution of 0.4 mg 1-ethyl-3 (3-dimethyl amino propyl) carbodimide hydrochloride (ECDI) in 2 ml distilled water has added dropwise and the reaction allowed to proceed at room temperature (22°C) overnight. The solution was dialysed against 3 changes of saline (5 litres). To estimate the number of moles of LHRH bound to tetanus toxoid 3nCi of  $^{131}\text{I}$  labelled LHRH was added into the reaction mixture. Using this procedure it is possible to determine the apparent incorporation of labelled LHRH on to the carrier protein by counting the quantity of  $^{131}\text{I}$  - LHRH in the solution before and after extensive dialysis. In this example 39 moles of LHRH were coupled to each mole of tetanus toxoid.

(b) Preparation of LHRH - tetanus toxoid immunogen using the gluteraldehyde coupling method.

An amount of 10 mg LHRH and 1200  $\mu\text{l}$  (13.6 mg) tetanus toxoid was dissolved in 34 ml water and 0.1 M NaOH added to give a final pH 8.0. Gluteraldehyde (4 ml of 25% v/v) was then added dropwise and the reaction allowed to proceed for 18 h at 25°C. The solution was dialysed as described in (a).

Calculation of  $^{131}\text{I}$  - LHRH activity before and after dialysis gave a value of 38 moles LHRH per mole tetanus toxoid.

(c) Preparation of LHRH - Protein A immunogen using the carbodiimide coupling method.

An amount of LHRH (10 mg in 10 ml water) was added to 500  $\mu\text{l}$  Protein A (50 mg) and 10 ml distilled water. The pH was adjusted to 6.5 with 0.01 M NaOH or HCl and 200 mg ECDI added in 10 ml distilled water.

The reaction was incubated at 25°C for 18 h as described in part (a).

Incorporation of  $^{131}\text{I}$  - LHRH to Protein A was estimated to yield a LHRH/protein A ratio of 1:36 after dialysis against three changes of physiological saline.

(d) Preparation of LHRH - Protein A immunogen using the gluteraldehyde coupling method.

10 An amount of 10 mg LHRH and 500  $\mu\text{l}$  (50 mg) protein A was dissolved in 34 ml distilled water and the pH adjusted to 8 using 0.01 M NaOH. To the mixture was added dropwise 4 ml of a 25% gluteraldehyde solution and the solution incubated at 15 25°C for 18 h. Following dialysis against three changes of saline the amount of incorporation of LHRH with protein A was calculated using the iodinated LHRH method as described previously in part A. Results showed that the apparent LHRH/protein A mole ratio was 20 1:34.

(e) Preparation of LHRH - Keyhole limpet haemocyanin (KLH) using carbodiimide as the coupling reagent.

To 10 mg LHRH and 20 mg KLH in 24 ml distilled 25 was added 0.01 M HCl to give a final pH 6.5. ECDI (10 ml of a 20 mg/ml solution) was then added to the mixture and incubated at 25°C for 18 h. The solution was dialysed against three changes of saline as described in part (a). Addition of  $^{131}\text{I}$  - LHRH to the 30 above solution indicated a LHRH/KLH mole ratio of 1:750 following dialysis.

(f) Preparation of LHRH - KLH using the gluteraldehyde as the coupling agent.

An amount of 5 mg LHRH and 10 mg KLH was dissolved in 12 ml distilled water, and the pH adjusted to 8.0 with 0.01 M NaOH. To this mixture 2 ml gluteraldehyde (25% w/v) was added dropwise and the solution incubated at 25°C for 18 h. The mixture was then dialysed against 3 changes of physiological saline as described in part (a).

10 Addition of  $^{131}$ -LHRH to the solution indicated that the apparent mole ratio of LHRH/KLH was 849.

(g) Diphtheria toxoid - LHRH

LHRH (200 mg) and diphtheria toxoid (137 ml, 219 mg) was dissolved in 10 ml distilled water and the pH of the solution adjusted to 6.0 with 0.1N NaOH.

15 A freshly prepared solution of 0.6g ECDI in 2 ml distilled water was added dropwise and the reaction allowed to proceed at room temperature (22°C) overnight. The solution was dialysed against 3 changes of saline (5 litres).

(h) Corynebacterium parvum - LHRH

LHRH (100 mg) and corynebacterium parvum (60 mg) were dissolved in 5 ml distilled water and the pH adjusted to 6.0 using 0.1 N NaOH. A freshly prepared solution of 0.2 g ECDI in 2 ml distilled water was added dropwise and the mixture incubated at room temperature overnight. - The solution was dialysed against 3 changes of saline (5 litres).

(i) Keyhole limpet haemocyanin - LHRH

30 LHRH (120 mg) and keyhole limpet haemocyanin (80 mg) were dissolved in 10 ml distilled water and the pH adjusted to 6.0 using 0.1N NaOH. A freshly prepared solution of 0.4g ECNT in 4 ml distilled water was added dropwise and the mixture incubated at room temperature overnight. The solution was dialysed against 3 changes of saline (5 litres).

The effectiveness of coupling, that is the moles of LHRH bound to carrier proteins 0.15 nCi  $^{125}\text{I}$ -LHRH was added to add of the above mixtures. After each reaction the percent bound  $^{125}\text{I}$ -LHRH was calculated based on the counts obtained before and after dialysis. Generally the effectiveness of coupling was good.

## B. RESULTS

### Experiment 1.

A flock of 42 Merino ram lambs were used in the study. The rams were selected by liveweight into 7 groups of 6 rams. (However during the course of the experiment some rams were lost; deaths were attributed to pneumonia). Treatments are shown below:

15

#### Group

1. Entire rams injected with the carrier proteins KLH, protein A and tetanus toxoid.
2. Rams surgically castrated at 10 weeks of age.
- 20 3. Rams immunized against a LHRH - tetanus toxoid conjugate.
4. Rams immunized against a LHRH-Protein A conjugate.
5. Rams immunized against a LHRH-KLH conjugate.
- 25 6. Rams immunized against a LHRH-HSA conjugate.
7. Rams immunized against LHRH-TT, LHRH - Protein A, and LHRH-KLH conjugates.

30

The dose, composition and time of injection of conjugates is shown in Table 1.

Each individual immunogen solution was emulsified with equal volumes of Freund's complete adjuvant. The ram lambs were then given 2 ml of the mixture in 4 separate subcutaneous sites in the back leg adjacent to the groin. The combined immunogens were injected with 8 ml of the emulsion. Booster injections were made using identical quantities of freund's incomplete adjuvant.

Blood samples were collected by venepuncture at two weeks following the second booster injection. The antibody titres for this experiment are illustrated in Table 3.

#### Experiment 2

A herd of 35 bull calves of predominantly Friesian breed were used for the experiment. The calves were raised on skim milk and weaned onto grass at 10 weeks of age. The bulls were divided into 5 groups of 7 animals using liveweight as the basis of selection (during the course of the experiment three bulls were lost due to pneumonia). Treatments are shown below.

25

<u>Group</u>	<u>Treatment</u>
1.	Entire bull calves were immunized against the carrier proteins, tetanus toxoid, keyhole limpet haemocyanin and protein A (n=6).

30



**TABLE 1**

**Composition, dose of immunogen and booster schedule  
for LHRH immunization in ram lambs**

5	Immunogen	Primary	First Booster	Second Booster	Third Booster
		10 weeks	Time after birth 18 weeks	26 weeks	34 weeks
10	Tetanus toxoid				
	- LHRH (Carb*)	400µg	400µg	400µg	400µg
15	Protein A				
	- LHRH (Carb)	900µg	900µg	900µg	900µg
20	KLH - LHRH (Carb)	900µg	900µg	900µg	900µg
	HSA-LHRH (Carb)	500µg	500µg	500µg	500µg
Tetanus toxoid-LHRH + Protein A-LHRH + KLH-LHRH:					
25	Tetanus toxoid				
	- LHRH (Carb) -	400µg	400µg	400µg	400µg
30	Protein A				
	- LHRH (Carb)	900µg	900µg	900µg	900µg
30	KLH - LHRH (Carb)	900µg	900µg	900µg	900µg

**TABLE 1 (continued)**  
**Composition, dose of immunogen and booster schedule**  
**for LHRH immunization in ram lambs**

5	Immunogen	Primary	First Booster	Second Booster	Third Booster
		Time after birth			
		10 weeks	18 weeks	26 weeks	34 weeks
10	Tetanus toxoid + KLH + Protein A:				
	Tetanus toxoid	400µg	400µg	400µg	400µg
15	KLH	400µg	400µg	400µg	400µg
	Protein A	900µg	900µg	900µg	900µg

\*Carbodiimide used to couple LHRH to carrier protein.

20

25

30

2. Bull calves surgically castrated at 14 weeks of age (n=6)
3. Bull calves immunized against a LHRH-HSA conjugate (n=6).
- 5 4. Bull calves immunized against a LHRH-tetanus toxoid conjugate (n=6).
5. Bull calves immunized against LHRH-coupled to tetanus toxoid, keyhole limpet haemocyanin and Protein A (n=6).

10

The composition, dose and immunization schedule for the experiment is outlined in Table 2.

For the immunization procedure the immunogen solution was emulsified in an equal volume of Freund's complete adjuvant. For all individual treatment  
15 groups a total of 2 ml of the emulsion solution was injected into 4-5 sites. For the combined immunogen group the bull calves were injected into 4-5 sites with 8 ml of the emulsion solution. Injections were  
20 given subcutaneously near the front shoulder and the brisket of the calves. For booster injections the immunogens were emulsified using Freund's incomplete adjuvant. Blood samples were collected into  
25 heparinized containers at two weeks after the second booster injection. The antibody titres for this experiment are given in Table 4.

A test was developed to study the social and sexual behaviour of the bulls. This involved exposing each bull calf individually to a non-oestrous female  
30 for 15 minutes and observing and recording the social

18

TABLE 2

Composition, dose of immunogens and booster schedule  
for LHRH immunization in bull calves

5	Immunogen	Primary	First Booster	Second Booster	Third Booster
		Time after birth			
		(14 weeks)	(22 weeks)	(30 weeks)	(38 weeks)
10	Tetanus toxoid				
	-LHRH (Carb)*	360µg	200µg	200µg	500µg
	-LHRH (Glut)**	360µg	200µg	200µg	500µg
	KLH - LHRH				
15	-(Carb)	360µg	200µg	200µg	500µg
	-(Glut)	360µg	200µg	200µg	500µg
	Protein A				
	-LHRH (Carb)	900µg	650µg	650µg	1500µg
20	-LHRH (Glut)	900µg	650µg	650µg	1500µg
	HSA - LHRH				
	-(Carb)	350µg	200µg	200µg	500µg
	-(Glut)	350µg	200µg	200µg	500µg
25					
	Tetanus toxoid-LHRH + KLH-LHRH + Protein A-LHRH:				
	Tetanus toxoid				
30	-LHRH (Carb)	360µg	200µg	200µg	500µg
	-LHRH (Glut)	360µg	200µg	200µg	500µg

**TABLE 2 (continued)**

**Composition, dose of immunogens and booster schedule  
for LHRH immunization in bull calves**

5	Immunogen	Primary	First Booster	Second Booster	Third Booster
		Time after birth (14 weeks) (22 weeks) (30 weeks) (38 weeks)			
10	KLH - LHRH				
	-(Carb)	360µg	200µg	200µg	500µg
	-(Glut)	360µg	200µg	200µg	500µg
	Protein A				
15	-LHRH (Carb)	900µg	650µg	650µg	1500µg
	-LHRH (Glut)	900µg	650µg	650µg	1500µg
	Tetanus toxoid + KLH + Protein A:				
20	Tetanus toxoid	360µg	200µg	200µg	500µg
	-KLH	360µg	200µg	200µg	500µg
	-Protein A	360µg	200µg	200µg	
	500µg				
25	* Carbodiimide used for coupling LHRH to carrier protein.				
	** Gluteraldehyde used for coupling LHRH to carrier protein.				

TABLE 3  
Antibody titres in ram lambs immunized against  
various LHRH conjugates

	Carrier Proteins KLH, TT and Protein A unconjugated	Keyhole limpet haemocyanin (KLH)	Tetanus toxoid (TT)	Human serum albumin (HSA)	Protein A	Combined KLH + TT and Protein A
5	<1:50	<1:50	<1:50	1:2000		1:1400
	<1:50	<1:50	<1:50	1:1700	<1:50	1:600
	<1:50	<1:50	1:1900	1:400	1:200	1:20000
10	<1:50	1:200	1:6000	<1:50	1:100	1:2500
	<1:50	1:100	1:3000			1:2000
	<1:50					1:6000
15						
	Mean					
	titre	<1:50	<1:150	1:2200	1:1050	1:130
						1:5146
	Range	NA	<1:50	<1:50-	<1:50-	1:600-
20		-1:200	1:6000	1:2000	1:200	1:20000
	No.	0	2/5	3/5	3/4	2/3
	animals responding over total number					6/6

21.

TABLE 4  
Antibody titres in individual bull calves immunized  
against various LHRH conjugates.

	Carrier proteins KLH, TT and Protein A, unconjugated	Tetanus toxoid (TT)	Human Serum Albumin (HSA)	Combined KLH + TT + Protein A
5	<1:10	1:500	1:170	1:150
	<1:10	<1:10	1:180	1:400
10	<1:10	1:25	<1:10	1:30
	<1:10	1:65	<1:10	1:50
	<1:10	<1:10	1:10	1:120
	<1:10	<1:10	<1:10	1:280
15	Mean	1:98	1:58	1:170
	Animals responding per total animals	0/6	2/6	6/6

and sexual behaviour of the animals. The results from this test are presented in Table 5.

TABLE 5

Effect of immunization Against LHRH on  
Social and Sexual Behaviour of Bulls  
After the Second Booster Injection

		TREATMENT				
10	Percentage of time in test engaging in:	Entire	Castrate	Comb.	TT	HSA
	Social-sexual inter- actions of a non- aggressive nature	1.01	0.31	0.61	0.68	1.41
	Sexual interactions	0.61	0.09	0.39	0.48	0.90
15	Social and sexual investigation	0.72	0.31	0.35	0.46	0.70
	Aggressive interaction	1.43	3.83	3.33	2.80	3.50

20 The antibody titres shown in Tables 3 and 4 for ram lambs and bull calves immunized respectively with various LHRH - conjugates and combinations thereof respectively, are depicted graphically in Figures 1 and 2.

25 In Figure 1, as expected, none of the ram lambs immunized with the carrier protein alone showed any immune response in the assay system. Lambs immunized with a single LHRH conjugate gave variable results in both antibody titre and the number of animals which responded. With KLH, only 40% of animals responded.

30 The mean titre of this response is 1:130. A similar result was obtained with protein A, where although 66% of animals tested gave an immune response (2 of the



animals in the experiment died from pneumonia), the mean titre of this response was 1:130. The best results using a single conjugate were obtained for tetanus toxoid, (TT) where 60% of animals showed an  
5 immune response with a mean titre of 1:2200.

The results obtained on immunization with a mixture of LHRH-TT, LHRH - Protein A and LHRH - KLH conjugates illustrate the efficacy of the present invention. All of the lambs tested gave a substantial  
10 immune response with a mean titre of 1:5146, with the antibody titre of individuals varying from 1:600 to 1:20,000. These results are clearly superior and are statistically significantly better ( $p < 0.05$  on  $\chi^2$  test of proportion of animals responding) to those obtained  
15 using a single LHRH - conjugate.

Similar results, including the same statistical significance to the above are shown in Figure 2 where all of the bull calves immunized with a mixture of LHRH coupled to tetanus toxoid, keyhole  
20 limpet haemocyanin and Protein A, showed a good immune response with a mean titre of 1:170, this being in contrast with the results obtained with LHRH coupled to a single carrier protein.

The behavioural data presented in Table 5 show  
25 that control animals spent more time involved in social and sexual behaviour and in social and sexual investigation than animals in the castrate, combination (LHRH individually coupled to KLH, TT and PA), and tetanus toxoid (TT coupled to LHRH)  
30 treatments. As expected, the castrated males spent the least time involved in social and sexual behaviour. The time spent involved in social and sexual behaviour by bulls in the combination treatment was more than that spent by the castrates but was less

than that spent by the controls. This suggests that active immunization against LHRH may influence the social and sexual behaviour of the bulls. Although the control animals spent less time involved in aggressive interactions than either the castrates or the combination animals it is encouraging to note that this attribute was similar for the castrates and for the animals in the combination group (it is possible that submissive test animals may be associated with aggressive interactions). Again, this suggests that the immunization may affect aggression. It is clear that there was a greater effect on the behaviour of the animals in the combination treatment than on the animals injected with tetanus toxoid-LHRH and HSA-LHRH conjugates.

### Experiment 3

A flock of 80 Merino X Corriedale rams at 5 months of age were used in this experiment. The rams (16 weeks of age) were allocated to 8 groups of 10 rams. One group were surgically castrated at 16 weeks and another group were left as entire animals. The remaining 60 rams were treated with a number of LHRH antigens.

Various LHRH - carrier protein conjugates were prepared as described previously. The antigens used were LHRH coupled separately to diphtheria toxoid, tetanus toxoid, keyhole limpet haemocyanin and corynebacterium parvum. Two other groups were injected with two different combinations of the individual LHRH complexes. Treatments are shown in Table 6, together with the total amounts of conjugates used. Rams were injected subcutaneously in the neck with these single LHRH carrier proteins using Freund's complete adjuvant.

Eight weeks following the booster injection pen tests were carried out on all animals to determine whether they were sexually active. This involved presenting each male with 4 oestrous females in an arena (5m X 5m) for 15 minutes and recording the bouts of anogenital investigation, bouts of courtship, number of mounts and number of ejaculations performed by the males. Six males were observed concurrently and all the animals were observed on the same day. The animals were kept in the testing arenas for 12 hours before the pen tests to allow them to become familiar with the testing situation. An animal was considered to be sexually active if he mounted and/or ejaculated during test.

TABLE 6

## TREATMENT AND DOSE RATES

Group	No. of Ewes	Treatment	Dose
20	1	Control	
	2	Castrate	
	3	LHRH-(TT)	1mg
	4	LHRH-(DT)	1mg
25	5	LHRH-(CP)	1mg
	6	LHRH-(KLH)	1mg (method 1)
	7	LHRH-DTT + LHRH-DT + LHRH-KLH	3mg
30	8	LHRH-DT + LHRH-CP + LHRH-KLH	3mg

TABLE 7

## LIVEWEIGHT CHANGES IN CONTROL CASTRATE AND TREATED LAMBS

5	Treatment Group	Time of testing				
		0	14 wks	17 wks	21 wks	27 wks
	Control	40.7	54.2	48.4	51.0	210.0
	Castrate	40.6	49.6	43.1	46.4	-
10	LHRH - TT	39.2	54.2	45.4	47.5	146.1
	LHRH - DT	39.3	53.9	45.6	48.7	83.3
	LHRH - CP	39.3	55.8	46.8	49.3	171.0
	LHRH - KLH	38.7	55.3	46.3	48.7	153.3
15	LHRH-TT+DT+KLH	38.3	54.7	46.5	48.6	71.0
	LHRH-DT+CP+KLH	38.7	55.5	44.9	48.6	55.0

20 Liveweights and testicular size (as determined using a series of calibrated beads) was measured every 4 weeks.

25 Antibody titre to LHRH were measured by both radioimmunoassay (RIA) and ELISA techniques. Plasma samples were serially diluted with phosphosaline buffer (0.05M, pH 7.6). The diluted samples (100 ul) were added to 100 ul bovine gamma globulin (3% 9w/v) and 100 ul of a buffer solution containing 2nCi <sup>125</sup>I-LHRH. The tubes were incubated overnight at 4°C. Free and bound LHRH were separated by adding 1 ml 21% polyethylene glycol 4000 and aspirating the supernatant after centrifugation. The tubes were counted for 1 minute in a clinigamma counter.

30 Liveweights of all rams are shown in Table 7. There were no significant differences among groups although the mean liveweight values for the immunised rams were higher than the castrated males but lower than the control rams. The mean testicular volumes for each treatment are shown in Table 8 and Figures 3 and 4.

27.

There was considerable variation with each individual antigen treatment. The largest decrease for individual carriers was observed in the rams immunised against LHRH coupled to diphtheria toxoid. The next best carriers were tetanus toxoid followed by keyhole limpet haemocyanin and corynebacterium parvum. The two different combinations of 3 separate antigens gave lower testes volumes than each of the individual antigens.

A similar trend in results was observed for the RIA results. The median titres were highest in the two groups with the combined antigens, followed by diphtheria toxoid, tetanus toxoid, keyhole limpet haemocyanin, corynebacterium parvum. The individual RIA and ELISA IgC results are shown in Table 9.

TABLE 8

TESTICULAR VOLUME IN CONTROL, CASTRATED  
AND IMMUNISED RAM LAMBS

Treatment Group	Time of testing				
	0	14 wks	17 wks	21 wks	27 wks
Control	160.0	168.0	183.5	187.0	210.0
Castrate	-	-	-	-	-
LHRH - TT	155.0	135.6	108.3	111.1	146.1
LHRH - DT	150.0	101.7	73.3	59.1	83.3
LHRH - CP	144.0	149.5	162.8	170.5	171.0
LHRH - KLH	153.3	133.5	136.1	127.2	153.3
LHRH-TT+DT+KLH	138.9	81.1	62.2	56.0	71.0
LHRH-DT+CP+KLH	141.1	77.5	67.8	48.3	55.0

TABLE 9

ANTIBODY LEVELS IN RAMS, LAMBS AS MEASURED  
BY ELISA AND RIA ASSAYS

Group No.	Sheep No.	Pre Booster Titre Elisa	1st Post Titre Elisa	2nd Post Titre	
				Elisa	RIA
1	701	0	0	0	<1:600
1	702	0	0	0	<1:600
1	703	0	0	0	<1:600
1	704	0	0	0	<1:600
1	705	0	0	0	<1:600
1	706	0	0	0	<1:600
1	707	0	0	0	<1:600
1	708	0	0	0	<1:600
1	709	0	0	0	<1:600
1	710	0	0	0	<1:600
2	(1958)	0	0	0	
2	713	0	0	0	
2	714	0	0	0	
2	715	0	0	0	
2	716	0	0	0	
2	717	0	0	0	
2	718	0	0	0	
2	719	0	0	0	
2	720	0	0	0	
2	753	0	0	0	
3	722	16	4	8	<1:600
3	723	4	32	128	1:6,200
3	724	0	4	4	1:810
3	726	0	16	8	<1:600
3	727	4	64	32	1:4,400
3	728	16	64	16	1:1,560
3	729	8	64	64	1:2,100
3	730	16	8	16	<1:600
3	731	16	8	8	1:870
4	768	32	1024	512	1:48,600
4	733	0	64	32	1:2,640
4	1912	32	512	512	1:48,600
4	735	16	128	128	1:5,400

TABLE 9 (CONTINUED)

4	775	-	128	256	1:28,200
4	737	32	64	64	1:3,780
4	738	32	8	16	1:1,320
4	739	64	256	64	1:2,940
4	741	8	32	0	1:1,020
5	742	0	8	64	<1:600
5	743	0	0	16	<1:600
5	744	0	0	2	<1:600
5	745	4	0	2	<1:600
5	746	0	0	4	<1:600
5	747	0	0	0	<1:600
5	748	16	16	32	<1:600
5	749	0	0	4	<1:600
5	750	-	0	4	<1:600
5	752	2	8	8	<1:600
6	721	-	256	256	
6	754	64	64	32	1:1,320
6	755	0	8	-	
6	756	8	16	16	<1:600
6	757	16	8	8	<1:600
6	758	4	16	16	<1:600
6	759	0	8	0	<1:600
6	760	16	32	16	1:2,160
6	761	64	128	1024	1:5,400
6	762	-	64	16	1:900
7	783	16	64	64	1:10,800
7	1910	16	64	128	1:7,200
7	785	32	32	32	1:2,400
7	786	-	128	128	1:9,900
7	787	64	32	128	1:41,900
7	788	64	32	128	1:3,660
7	1864	0	0	0	1:600
7	790	16	0	4	<1:600
7	791	- 32	8	4	1:1,200
7	792	32	32	512	1:27,600
8	793	16	64	64	1:5,600
8	794	-	512	256	1:3,660
8	795	4	8	32	1:1,800
8	796	0	128	128	1:12,600
8	797	512	256	256	1:22,200
8	798	8	32	32	1:9,000
8	800	32	32	32	1:1,440
8	772	4096	1024	256	1:44,000

TABLE 10

EFFECT OF ACTIVE IMMUNISATION AGAINST LHRH  
ON THE PROPORTION OF ANIMALS MOUNTING AND/OR EJACULATING

Treatment Group	Proportion of Animals Mounting and/or ejaculating
Control	10/10
Castrate	0/9
LHRH - TT	2/9
LHRH - DT	1/9
LHRH - CP	5/9
LHRH - KLH	3/10
LHRH - TT + DT + KLH	0/10
LHRH - DT + CP + KLH	0/8



In general those rams with high RIA titres had elevated titres as measured by the ELISA assay. However discrepancies were found in individual rams, in some instances high RIA titres were found in samples with low IgC titres. These results suggest that the RIA assay is monitoring a higher number of antibody subclasses than the more specific IgG ELISA assay.

The proportion of rams in each group that were sexually active at 2 months after the booster injection are shown in Table 10. Ten out of a total of 10 rams in the control group were showed sexual activity. The best single antigen was diphtheria toxoid with 1 of 9 rams sexually active. The order of effectiveness was diphtheria toxoid, tetanus toxoid, keyhole limpet haemocyanin and corynebacterium parvum. The two groups with the combined antigens were not sexually active. For those rams given a combination of TT, DT and KLH coupled to LHRH there were no rams active out of a total of 10 rams. Similar results were obtained using a different combination of carriers, DT CP and KLH where again there were no rams active out of a total of 8 rams (two rams died during the trial). None of the castrated rams were sexually active.

These results indicate that a combination of various carrier protein gives a better physiological response than each individual antigen given separately. This effect was observed for testicular size, RIA titres and for the proportion of animals that were sexually active. Overall these results indicate combinations of LHRH antigens result in a better immune response compared with individual antigens.

Other aspects of the present invention and modifications and variations thereto, will become apparent to those skilled in the art on reading this specification, and all such other aspects and modifications and variations are considered as included within the scope of the present invention.

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CLAIMS

1. A composition for the production in animals of antibodies specific for LHRH, said composition comprising two or more different carriers individually coupled to LHRH or analogues of LHRH, in amounts sufficient to elicit an immune response against LHRH.
2. A composition as claimed in claim 1, wherein at least two of the carriers are carrier proteins.
3. A composition as claimed in claim 2 wherein at least one of the carrier proteins is Diptheria toxoid.
4. A composition as claimed in any one of claims 1-3 wherein the composition further comprises an adjuvant.
5. A composition as claimed in claim 4 wherein the adjuvant selected is such the composition causes no inflammation.
6. A method for the suppression of male social and sexual behaviour of domestic animals, comprising the administration of a composition as claimed in any one of claims 1 to 5.
7. A method for the suppression of ovulation and oestrous cyclicity of female domestic animals comprising the administration of a composition as claimed in any one of claims 1 to 5.
8. A method as claimed in claim 6 or wherein there are two or more administrations of said compositions.

9. A method as claimed in any one of claims 6 to 8, where the animals are selected from dogs, cats, cattle, sheep, donkeys, rabbits and deer.

10. A composition for the production in animals of antibodies specific to a substance which when conjugated to a carrier is capable of eliciting an immune response in the animal wherein the composition comprises a mixture of the substance conjugated to at least two different carriers.

1/4

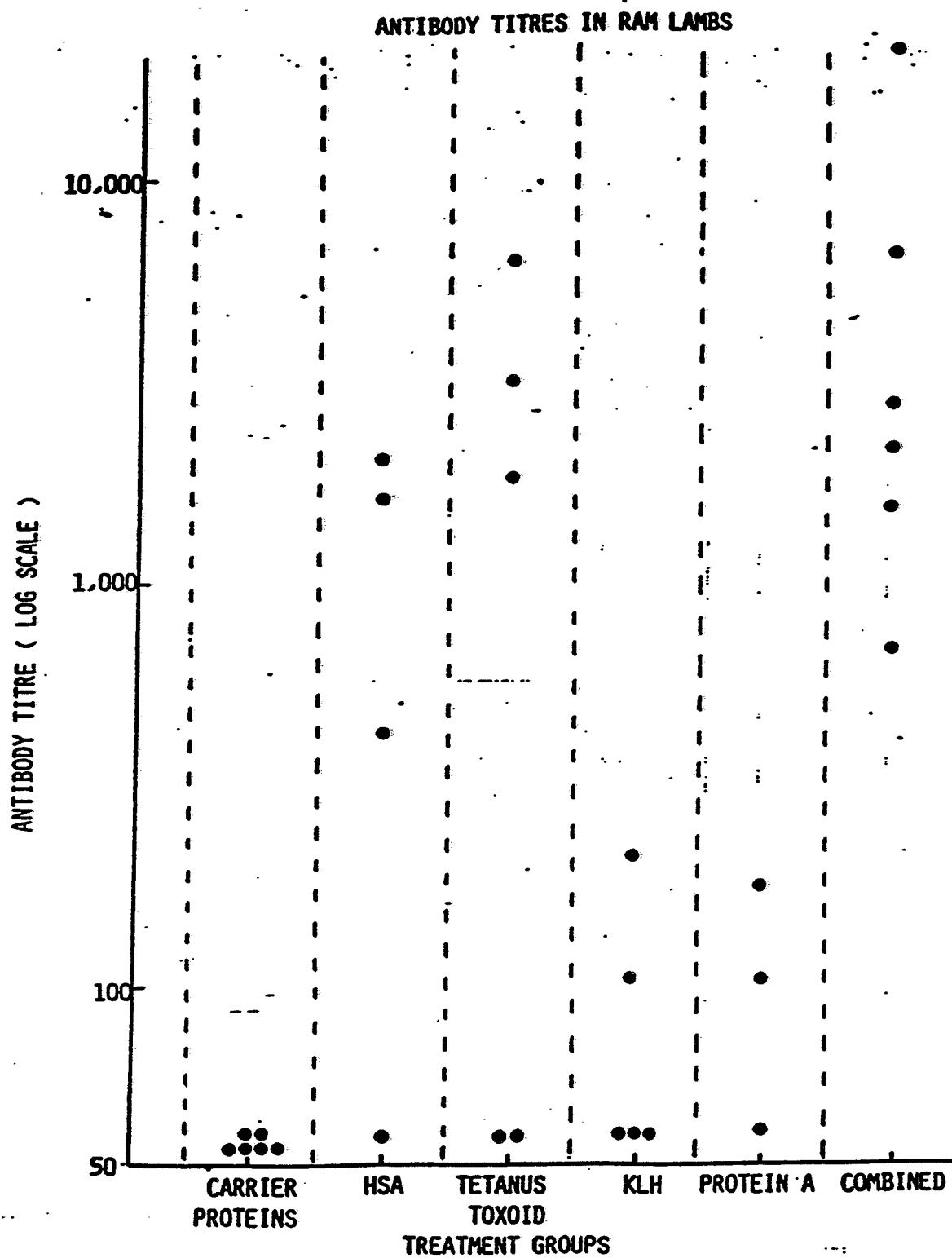


FIGURE 1

2/4

## ANTIBODY TITRES IN BULL CALVES

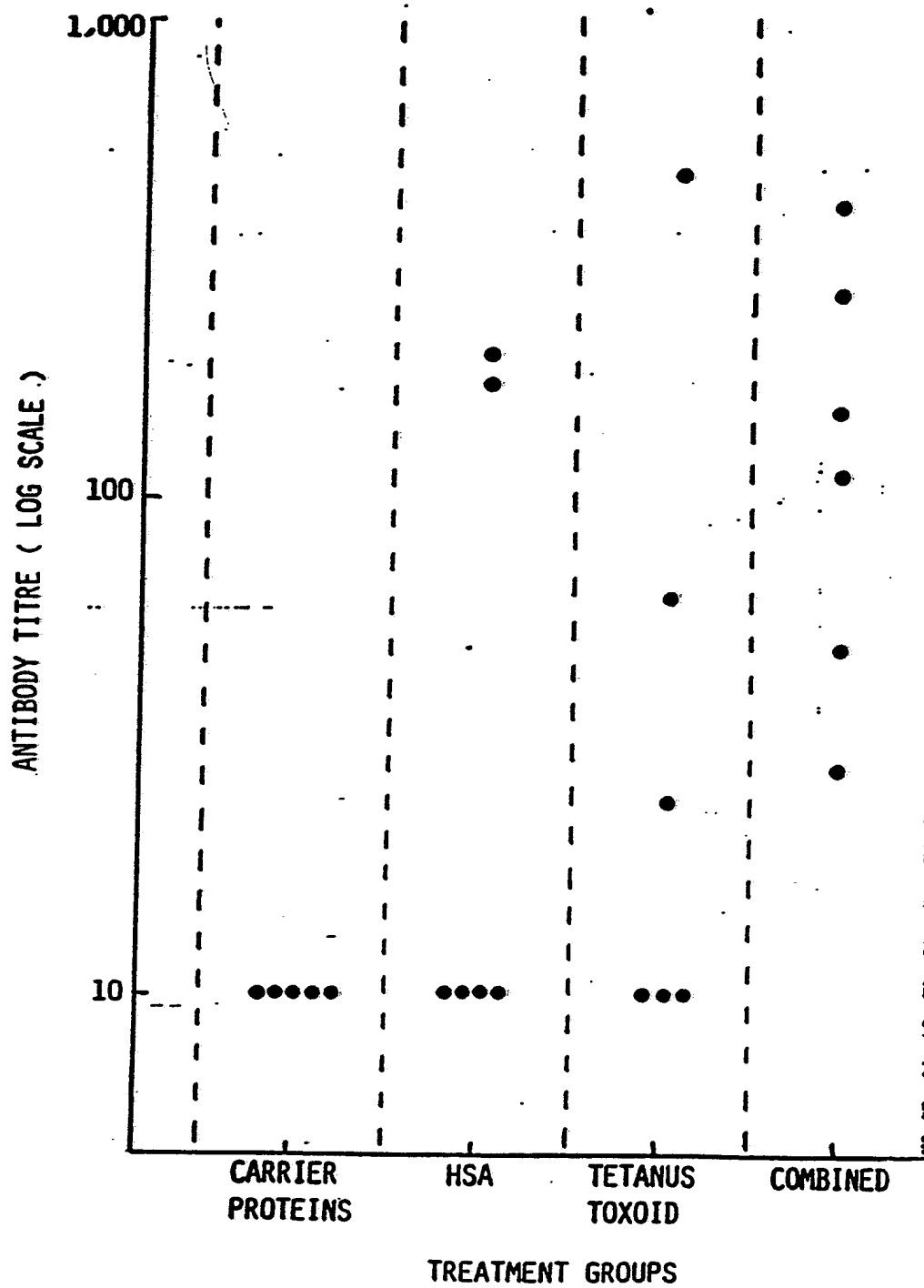


FIGURE 2



3/4

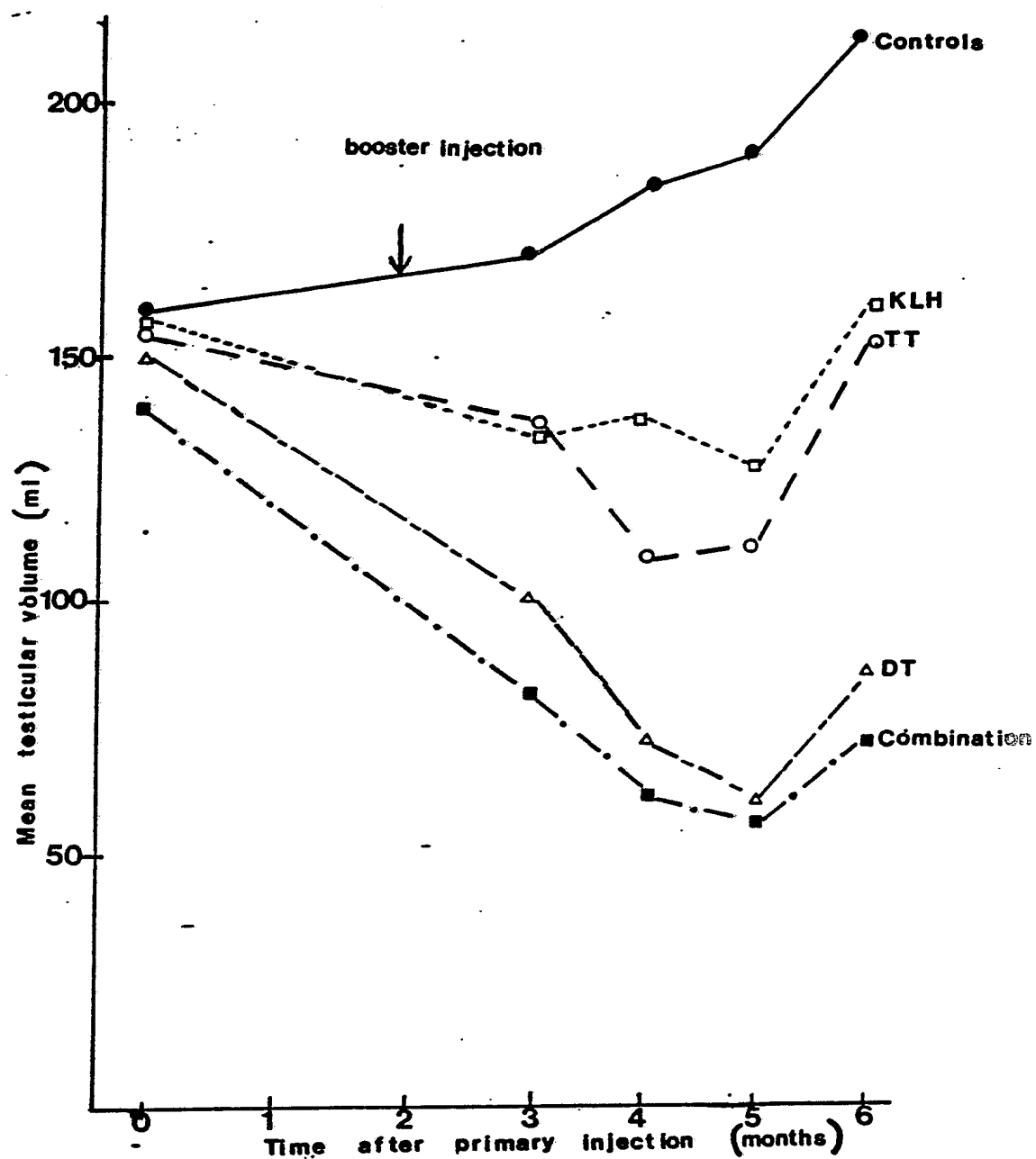


FIGURE 3

4/4

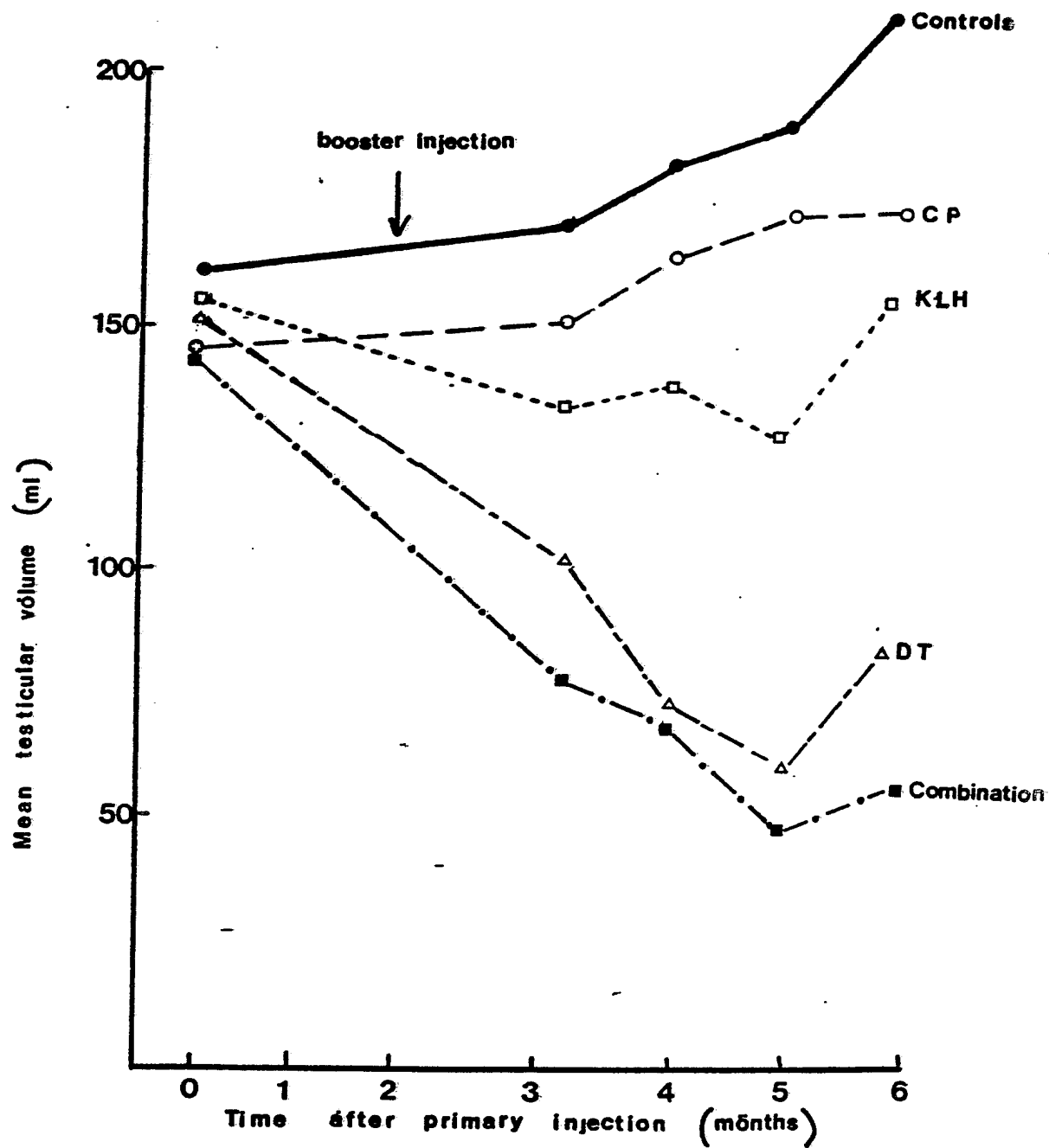


FIGURE 4

# INTERNATIONAL SEARCH REPORT

International Application No PCT/AU 87/00199

## I. CLASSIFICATION OF SUBJECT MATTER (If several classification symbols apply, indicate all.)

According to International Patent Classification (IPC) or to both National Classification and IPC

Int. Cl.<sup>4</sup> A61K 37/43, 37/24

## II. FIELDS SEARCHED

Minimum Documentation Searched<sup>7</sup>

Classification System

Classification Symbols

IPC

A61K 37/24, 37/43

Documentation Searched other than Minimum Documentation  
to the Extent that such Documents are Included in the Fields Searched<sup>8</sup>

AU: IPC as above; Australian Classification 87.16

## III. DOCUMENTS CONSIDERED TO BE RELEVANT<sup>9</sup>

Category<sup>10</sup> Citation of Document, <sup>11</sup> with indication, where appropriate, of the relevant passages <sup>12</sup> Relevant to Claim No. <sup>13</sup>

- |   |  |       |
|---|--|-------|
| A | Chemical Abstracts, Volume 98, No.17, issued 1983, April 25 (Columbus, Ohio, U.S.A.), Donna L. Vogel et al, 'Sertoli cell maturation is impaired by neonatal passive immunization with antiserum to LHRH', abstract No. 137896q. | (1-9) |
|---|--|-------|

### \* Special categories of cited documents: <sup>14</sup>

- "A" document defining the general state of the art which is not considered to be of particular relevance
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"Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

## IV. CERTIFICATION

Date of the Actual Completion of the International Search

11 September 1987 (11.09.87)

Date of Mailing of this International Search Report

(25.09.87) 25 SEPTEMBER 1987

International Searching Authority

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